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Integrated microfluidic magnetic immunosensor for quantification of human serum IgG antibodies to *Helicobacter pylori*☆

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ABSTRACT

In this paper, we have developed and characterized a microfluidic magnetic immunosensor coupled to a gold electrode for the rapid and sensitive quantification of human serum IgG antibodies to *Helicobacter pylori*. This microorganism cause peptic ulcers and chronic gastritis, affecting around the 10% of the world population. The sensor was completely automated and the antibodies detection in serum samples was carried out using a non-competitive immunoassay based on the use of purified *H. pylori* antigens that are immobilized on magnetic microspheres 3-aminopropyl-modified. The magnetic microbeads were injected into microchannel devices and manipulated for an external removable magnet. The IgG antibodies in human serum sample are allowed to react immunologically with the immobilized antigens, and the bounded antibodies are quantified by alkaline phosphatase (AP) enzyme-labeled second antibodies specific to human IgG. The *p*-aminophenyl phosphate (*p*-APP) was converted to *p*-aminophenol (*p*-AP) by AP and an electroactive product was detected on gold layer electrode at 0.250 V. The response current obtained from the product of enzymatic reaction is directly proportional to the activity of the enzyme and, consequently, to the amount of IgG antibodies to *H. pylori* in serum samples. The electrochemical detection can be done within 1 min and total assay time was 25 min. The calculated detection limits for electrochemical detection and the ELISA procedure were 0.37 and 2.1 U mL^{-1}, respectively, and the within- and between-assay coefficients of variation were below 5%. Our results indicate the potential usefulness of our fabricated microbiochip for the early assessment of human serum immunoglobulin G (IgG) antibodies to *H. pylori*.

1. Introduction

The Gram-negative bacterium *Helicobacter pylori* is the most important etiological agent of chronic active type B gastritis and peptic ulcer diseases. In addition, infection with this organism is a recognized risk factor in the development of gastric mucosa-associated lymphoid tissue lymphoma and adenocarcinoma [1]. Although in most of the cases, the infection does not result in clinical symptoms, the Public Health relevance of this illness is high. Chronic gastritis and peptic ulcer are very common diseases across the populations. Moreover, gastric cancer remains in the second place among the causes of cancer deaths worldwide [2].

The goal of *H. pylori* treatment is the complete elimination of this microorganism. The combination of two or more antimicrobial agents is very important because it increases the rates of cure and reduces the risk of selecting resistant *H. pylori*. The chief antimicrobial agents used in these treatments are amoxicillin, clarithromycin, metronidazole, tetracycline, and bismuth [3].

The concern regarding the diagnosis of this microorganism, during the past years, has been the subject of several reviews and numerous original articles that reported the use of the different invasive methods as endoscopy with biopsies for histology, culture and a rapid urease test and the noninvasive tests including serological tests and the urea breath test [4,5]. Some noninvasive tests are based on serological procedures that detect IgG against *H. pylori* in human serum. Circulating anti-*H. pylori* IgG antibodies have proved to be of considerable value in the diagnosis of active infection due to the reliable correlation between the presence of the antibodies and gastric mucosal colonization [6,7]. Common serum IgG measurements are carried out using enzyme-linked immunosorbent assay (ELISA) [8].

Heterogeneous enzyme immunoassays, coupled with flow injection (FI) system and amperometric detection, represent a powerful analytical tool for the determination of low levels of many analytes such as antibodies, hormones, drugs, tumor markers, and viruses [9].

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Regarding the immunoassay field, various microfluidic devices have been used to adopt the conventional (ELISA) at microscale, including glass chips with embedded microbeads [10] or polydimethylsiloxane chips with microfluidic channels [11]. The manipulation of fluids in channels with dimensions of tens of micrometres microfluidics has emerged as a distinct new field. Micrometre-scale analytical devices are more attractive than their macroscale counterparts for various reasons [12–15]. Which include that microfluidic biosensors allow the application of Green Chemistry because they are an efficient method for miniaturisation, use smaller volumes of reagents and samples [16], improved efficiency with regard to sample size, response time, cost, analytical performance, process control, integration, throughput and automation [17].

Immunoaffinity-based separation techniques have been used to separate analytes of interest from complex biological samples based on the selective binding of antibodies to their respective antigens [18]. Immunoaffinity microfluidic devices employing antibodies as immobilized ligands and may be applied to capture and concentrate target analytes from small volumes (microliters and below) of biological matrices [19].

The microparticles are an interesting tool as solid supports for microfluidic immunoassay. Compared to microtiter wells commonly used in immunoassays, the reactive surface area versus solution volume ratio is large and the diffusion distances are reduced in capillaries and microchannels [20]. In addition, a large number of analyte molecules are bound within a small volume, allowing a sensitive detection [21,22].

In this work, we coupled a microfluidic magnetic immunosensor to a gold electrode for the rapid and sensitive quantification of human serum IgG antibodies to *H. pylori*. Antibodies detection in serum samples was carried out using a non-competitive immunoassay based on the use of purified *H. pylori* antigens that are immobilized on magnetic microspheres 3-aminopropyl-modified. The magnetic microbeads were injected into microchannel devices and manipulated for an external removable magnet. The IgG antibodies in human serum sample are allowed to react immunologically with the immobilized antigens, and the bounded antibodies are quantified by alkaline phosphatase (AP) enzyme-labeled second antibodies specific to human IgG. The *p*-aminophenyl phosphate (*p*-APP) was converted to *p*-aminophenol (*p*-AP) by AP, whose back electrochemical oxidation was detected on gold electrode at 0.250 V. The response current obtained from the product of enzymatic reaction is directly proportional to the activity of the enzyme and, consequently, to the amount of IgG antibodies to *H. pylori* in serum samples. Our results indicated that the specific IgG antibodies to *H. pylori* were successfully assayed using our fabricated microbiochip, further demonstrating the utility of our system as a Serological diagnostic tool.

2. Materials and methods

2.1. Reagents and solutions

All reagents used were of analytical reagent grade. AP enzyme-labeled second antibodies specific to human γ-chain was purchased from Sigma Chemical (St. Louis, MO, USA). Glutaraldehyde (25% aqueous solution) was purchased from Merck, Darmstadt. The 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP) was purchased from Flucka Chemie (Steinheim, Switzerland). The microparticles, magnetic, amino functionalized were purchased by Fluka, Buchs/Schweiz, USA. All other reagents employed were of analytical grade and used without further purifications. Aqueous solutions were prepared using purified water from a Milli-Q system.

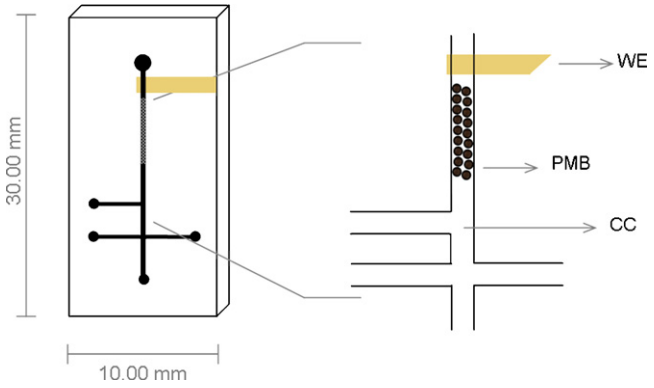

Fig. 1. Schematic representation of microfluidic immunosensor. WE: gold working electrode, PMB: paramagnetic beads, and CC: central channel. All measurements are given in millimeters.

The ELISA test kit for the quantitative determination of *H. pylori*-specific IgG class antibodies was purchased from EQUIPAR Diagnostici (Rome, Italy) and was used in accordance with the manufacturer's instructions [23].

2.2. Flow-through reactor/detector unit

The main body of the sensor was made of Plexiglas. Fig. 1 illustrates the design of the flow-through chamber containing the microfluidic immunosensor and the detector system. The gold layer electrode of 80 nm thickness was deposited at central channel (CC) by sputtering (SPI-Module Sputter Coater with Etch mode, Structure probe Inc., West Chester, PA) and the gold thickness electrode was measured using a Quartz Crystal Thickness Monitor model 12161 (Structure probe Inc., West Chester, PA) [24,25]. The diameter of the CC and the accessory channels was 75 μm. The electrode was cleaned using cyclic voltammetry in 0.5 M sulphuric acid by three-fold cycling in the potential range between −300 and 1700 mV at 100 mV s^{-1} scan rate.

All solutions and reagents were conditioned to 37 °C before the experiment, using a laboratory water bath Vicking Mason II (Vicking SRL, Argentina). Amperometric detection was performed using the BAS LC-4 C (Bioanalytical Systems, West Lafayette, IN, USA). The BAS 100B electrochemical analyzer (Bioanalytical Systems) was used for cyclic voltammetric analysis. The potential applied to the gold electrode was 250 V versus the Ag/AgCl wire pseudo-reference electrode and a Pt wire was the counter-electrode. At this potential, a catalytic current was well established. Pumps (Baby Bee Syringe Pump, Bioanalytical Systems) were used for pumping, sample introduction, and stopping flow.

All pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.). Absorbance was determined with a Bio-Rad Benchmark microplate reader (Japan) and Beckman DU 520 General UV/vis spectrophotometer.

2.3. H. pylori-specific IgG antibody immunoassay

A series of standards that covered the clinically relevant range (0–100 U mL^{-1}) were supplied with the ELISA test kit. A standard curve for the spectrophotometric procedure was produced by following the manufacturer's protocol [23]. Concentrations of *H. pylori*-specific IgG antibody were detected spectrophotometrically by measuring absorbance changes at 450 nm.

2.4. Preparation of the H. pylori antigens

The antigens were prepared from a sonicate *H. pylori* culture strain. The *H. pylori* were grown on blood agar plates at 37 °C or 3 days and then harvested, washed, and resuspended in 0.01 M phosphate-buffered saline (PBS, pH 7.2). This preparation was subjected to sonication. The sonic amplitude level was set at 20, and the machine was operated using four cycles of 60 s regulated alternatively. The sonicated preparation was centrifuged at 1000 × *g* for 10 min, and the supernatant was stored in the 0.01 M PBS (pH 7.2), at −20 °C between uses.

2.5. Synthesis of p-APP

Synthesis of *p*-APP by catalytic hydrogenation of pNPP was performed using the procedure of Ref. [26] with the following modifications. In a 100 ml glass hydrogenation vessel, 2.00 g of pNPP was dissolved in 30 ml of 50% ethanol containing 0.11 g of 10% palladium on charcoal catalyst. The hydrogenation reaction was conducted overnight at room temperature at an initial pressure of 1.3 atm. The resultant mixture was filtered on a buchner funnel to remove the catalyst and the volume of solvent was reduced to 10 ml using a rotary evaporator. The oily residue was diluted to 20 ml with distilled, deionised water and clarified by filtration. Cold ethanol (20 ml, 4 °C) was added to the filtrate and the precipitated product was recovered by filtration, dried under vacuum, and stored at −10 °C. The *p*-APP product was greater than 98% pure as determined by NMR and electrochemical methods.

2.6. Immobilization of purified antigen of H. pylori on paramagnetic beads

Purified antigen of *H. pylori* was immobilized on magnetic microbeads modified with amino groups in an Eppendorf tube. An aliquot of 100 μL of water suspension of magnetic beads modified with aminopolystyrene were washed with 1.0 ml of PBS buffer pH 7.2 for three times. The pellet was suspended in 1.0 ml of an aqueous solution of 5% (w/w) glutaraldehyde at pH 10.00 (0.20 M carbonate) with continuous mixing for 2 h at room temperature. After three washes with PBS buffer pH 7.2 to remove the excess of glutaraldehyde, 500 μL of antigen preparation (100 μg mL^{-1} 0.01 M PBS, pH 7.2) was coupled to the residual aldehyde groups with continuous mixing for 12 h at 4 °C. The immobilized antigen on microbeads was finally washed with PBS and stored in the same buffer (in a final volume of 250 μL) at 5 °C. Immobilized antibody preparation was perfectly stable for at least 1 month.

2.7. Procedure for electrochemical immunosensor

This method was applied in the determination of IgG antibodies to *H. pylori* in 38 human serum samples. Firstly the antigen modified magnetic microbeads were pretreated with desorption buffer (0.1 M glycine–HCl, pH 2) and rinsed with 0.01 M PBS (pH 7.2). The unspecific binding was blocked by 10 min treatment at 37 °C with 3% skim milk in a 0.01 M phosphate buffer saline (PBS), pH 7.2 and finally washed with 0.01 M PBS buffer (pH 7.2) and stored in 250 μL of the same buffer. After this step, a suspension of microbeads (10 μL in 100 μL of PBS buffer) was injected using syringe pumps at a flow rate of 2.5 μL min^{-1} for 4 min and were maintained in a fixed position in the central channel, near the gold electrode by the action of a magnetic field generated by a removable, external magnet which was not removed during the full experiment. The carrier buffer was 0.01 M PBS, pH 7.2. The serum samples were first diluted 100-fold with 0.01 M PBS (pH 7.2) and then, they were injected into the PBS carrier stream at a flow rate of 2.5 μL min^{-1} for 5 min. The IgG specific antibodies to *H. pylori* present in the serum sample reacted immunologically with antigens of *H. pylori* immobilized over magnetic beads. The microfluidic device was washed with 0.01 M PBS (pH 7.2) at a flow rate of 2.5 μL min^{-1} for 4 min to remove the excess of sample. The bound antibodies were quantified using an alkaline phosphatase enzyme-labeled second antibodies specific to human IgG (dilution of 1/2000 in 0.01 M PBS, pH 7.2) injected at a flow rate of 2.5 μL min^{-1} for 5 min. Then, the microfluidic device was washed free of any traces of unbound enzyme conjugate with 0.01 M PBS (pH 7.2). Diethanolamine buffer (DEA) (100 mM diethanolamine, 50 mM KCl, 1 mM $MgCl_2$, pH 9.6) was used to prepare the *p*-APP solution (2.7 mM *p*-APP in a DEA buffer, pH 9.6), which was injected into the carrier stream at a flow rate of 2.5 μL min^{-1} for 1 min and the enzymatic product *p*-aminophenol (*p*-AP) was detected on the gold surface electrode. For the next analysis, the immunosensor was exposed to a flow of desorption buffer (0.1 M glycine–HCl, pH 2) at a flow rate of 2.5 μL min^{-1} for 5 min and then washed with PBS, pH 7.2. Our device was reused for five determinations without the loss of sensibility.

A standard curve for the electrochemical procedure was produced following our protocol with a series of standards that covered the clinically relevant range (0–100 U mL^{-1}) supplied with the ELISA test kit. Electrochemical measurements were performed at 37 °C and the resulting anodic current was measured. The stock solution of *p*-APP was prepared freshly before the experiment and stored under the exclusion of light for the duration of the experiment.

3. Results and discussion

3.1. Electrochemical study of p-AP

The electrochemical behaviour of the hydrolysis products *p*-AP of the enzyme substrates *p*-APP, was examined by cyclic voltammetry (CV) at gold electrode (Fig. 2). A cyclic voltammetric study of 1.0 × 10^{-3} mol L^{-1} of *p*-AP in DEA buffer (pH 9.6), was performed by scanning the potential from −300 to 500 mV versus Ag/AgCl. CV showed well-defined anodic and corresponding cathodic peak, which corresponds to the transformation of *p*-AP to *p*-benzoquinoneimine (QI) and vice versa within a quasi-reversible two-electron process. A peak current ratio (IC1/IA1) of nearly unity, particularly during the recycling of potential, can be considered as criteria for the stability of QI produced at the surface of electrode under the experimental conditions.

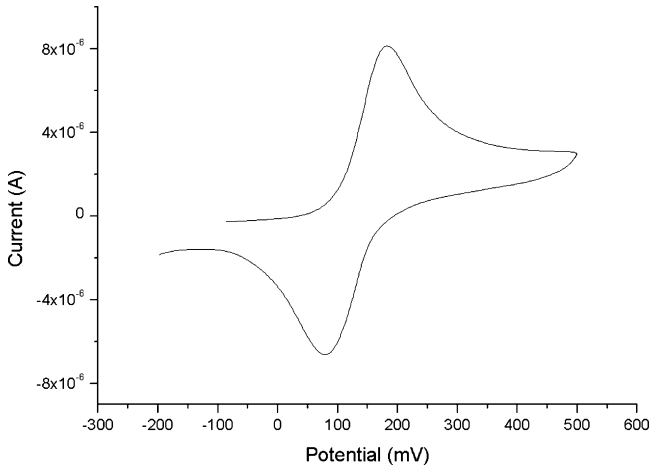

Fig. 2. Cyclic voltammogram of *p*-AP in aqueous solution containing 1 mM of 4-TBC in 0.10 M phosphate–citrate buffer, pH 5.0 with a gold electrode. Scan rate: 100 mV s^{-1}.

3.2. Optimum conditions for the immune reactions and the determination of enzymatic products

Microfluidic control systems are essential for the control of a minute volume of fluid because of their rapid and precise control features, therefore, in our microbiochip, all reactions and washing procedures were performed using a syringe pump. The flow rates of the sample and reagent have an effect on the reaction efficiencies of the antigen–antibody interactions and unlike conventional immunoassays, samples and reagents in our system are continuously flowing through the microbiochips. Therefore, it is very important to consider flow rate when designing microfluidic biosensors [27].

In a flow system, the flow rate of the solution passing through the microfluidic device channel is the main factor, affecting the dispersion of the analyte, yield of the reaction between the antigen immobilized on microbeads and the antibody present in the sample (one of the most critical process for the determination) and response of the electrochemical detector. So the optimization of flow rate is necessary. The optimal flow rate was determined by analyzing a standard of 20 U mL^{-1} *H. pylori*-specific antibody at different flow rates and evaluating the current generated during the immune reaction. As shown in Fig. 3, flow rates from 1 to 2.5 μL min^{-1} had little effect on antigen–antibody reaction. Conversely, when the flow rate exceeded 4 μL min^{-1}, the signal was dramatically reduced. Taking into account the size of the response and analysis time for each sample, 2.5 μL min^{-1} was chosen for sample injection and washing buffer injection, respectively.

The sample size was studied in the range 1–25 μL. Sensitivity is almost quadruplicated in the range between 1 and 13 μL. Insignificant differences were obtained for greater sample size. A sample size of 12.5 μL was used.

The rate of enzymatic response under flow conditions was studied in the pH range 8–10 and showed a maximum value of activity at pH 9.6. The pH value used was 9.6 in DEA buffer. The effect of varying *p*-APP concentration from 0.1 to 5 mM on the enzymatic response was evaluated. The optimum *p*-APP concentration found was 2.7 mM. That concentration was then used.

3.3. Quantitative test for the detection of H. pylori-specific IgG antibodies in the microfluidic immunosensor

Under the selected conditions described above, the electrochemical response of the enzymatic product is proportional to the concentration of *H. pylori*-specific IgG antibodies in serum. As shown in Table 1, which summarizes the complete analytical procedure required for the *H. pylori* immunoassay using our system, the total time required for the immunoassay was found to be approximately 25 min.

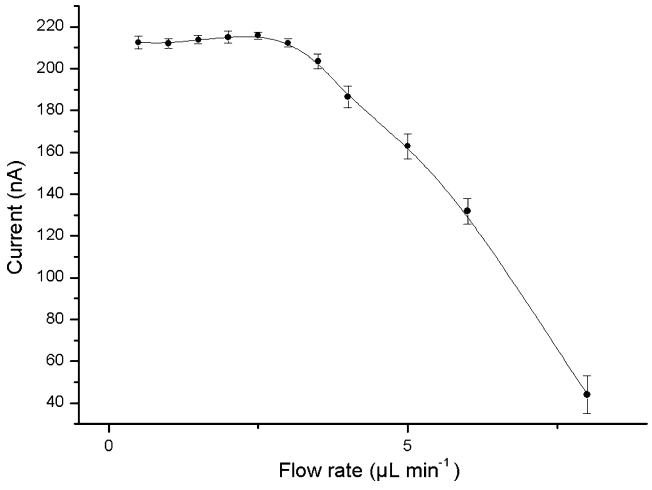

Fig. 3. Effect of flow rate analyzing a 100 U mL^{-1} *H. pylori*-specific antibodies standard at different flow rates.

Table 1
Sequences required for the *H. pylori*-specific IgG antibody immunoassay.

Sequence	Condition	Time
Injection of magnetic beads	10 μL of modified magnetic beads 2.5 μL min^{-1}	4 min
Washing buffer	Flow rate: 2.5 μL min^{-1} (PBS, pH 7.2)	2 min
Serum samples	Diluted 100-fold 2.5 μL min^{-1}	5 min
Washing buffer	Flow rate: 2.5 μL min^{-1} (PBS, pH 7.2)	4 min
Enzyme conjugated	AP-conjugated (dilution of 1/2000) 2.5 μL min^{-1}	5 min
Washing buffer	Flow rate: 2.5 μL min^{-1} (PBS, pH 7.2)	4 min
Substrate	2.5 μL 2.7 mM *p*-APP in a DEA buffer, pH 9.6	1 min
Signal analysis	LC-4C amperometric detector, 0.10 V	1 min

A linear calibration curve to predict the concentration of *H. pylori*-specific IgG antibodies in serum was produced within the range of 0–100 U mL^{-1}. The linear regression equation was $i = 0.115 + 2.137^{*}C_{H.\ pylori}$, with the linear regression coefficient $r = 0.999$. The coefficient of variation (CV) for the determination of 20 U mL^{-1} *H. pylori*-specific antibody was below 3.2% (six replicates). These values demonstrate that our microfluidic immunosensor can be used to quantify the amount of *H. pylori*-specific IgG antibodies in unknown samples.

The ELISA procedure was also carried out as described, absorbance changes were plotted against the corresponding *H. pylori*-specific IgG antibody concentration and a calibration curve was constructed. The linear regression equation was $A = 0.137 + 0.029^{*}C_{H.\ pylori}$, with the linear relation coefficient $r = 0.995$, the CV for the determination of 20 U mL^{-1} *H. pylori*-specific antibodies was 4.7% (six replicates).

The detection limit (dl) was considered to be the concentration that gives a signal three times the standard deviation (SD) of the blank. For electrochemical detection and EIA procedure the dl was 0.37 and 2.1 U mL^{-1}, respectively. This result shows that electrochemical detection was more sensitive than spectrophotometric method. The sensitivity (S) is defined as the slope of the regression line signal versus concentration, S for electrochemical detection and ELISA procedure was 2.137 nA/U mL^{-1} and 0.029 Abs/U mL^{-1}, respectively.

The precision of the electrochemical assay was checked with control serum at 20, 50 and 100 U mL^{-1} *H. pylori*-specific antibody concentrations. The within-assay precision was tested with five measurements in the same run for each serum. These series of analyses were repeated for three consecutive days in order to estimate the between-assay precision. The results obtained are presented in Table 2. The *H. pylori* assay showed good precision; the CV within-assay values were below 3.2% and the between-assay values were

Table 2
Within-assay precision (five measurements in the same run for each control serum) and between-assay precision (five measurements for each control serum, repeated for three consecutive days).

Control sera[a]	Within-assay		Between-assay	
	Mean	CV (%)	Mean	CV (%)
20 U mL^{-1}	20.37	3.11	21.09	4.76
50 U mL^{-1}	50.08	2.18	49.04	3.13
100 U mL^{-1}	100.82	2.98	99.23	3.56

[a] U mL^{-1} *H. pylori*-specific antibodies.

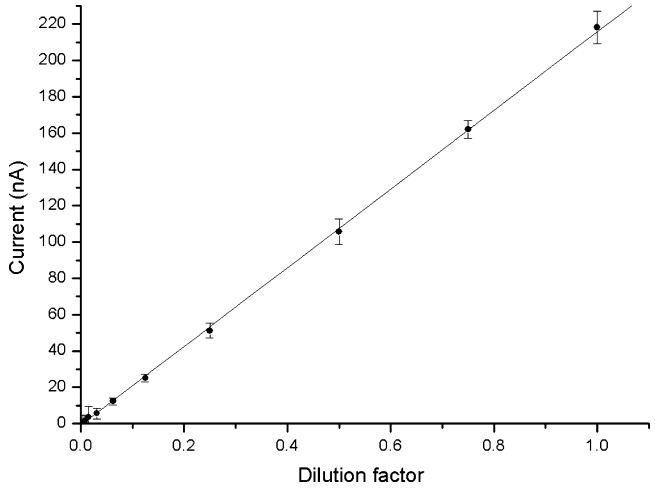

Fig. 4. Dilution test results for 100 U mL^{-1} *H. pylori*-specific antibodies. Each value of current (nA) is based on five determinations.

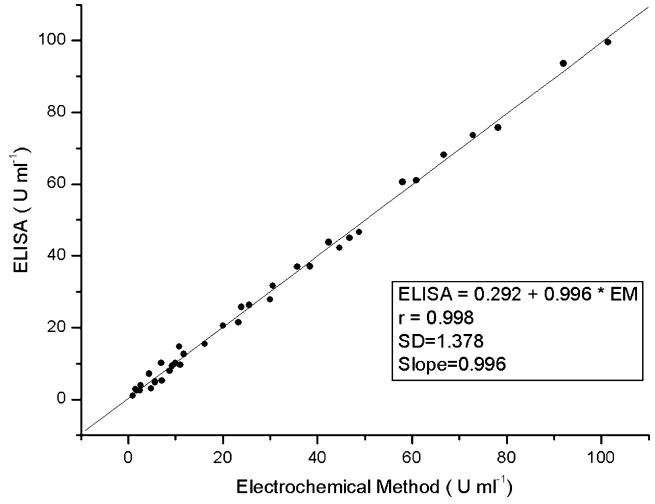

Fig. 5. Correlation between proposed method and commercial photometric assays.

below 5%. The accuracy was tested with dilution and recovery tests. A dilution test was performed with 100 U mL^{-1} *H. pylori*-specific antibodies control sera with 0.01 M PBS, pH 7.2 (Fig. 4).

The total assay time, including all injections and detection steps for the *H. pylori*-specific IgG antibodies measurements was less than 25 min (much less than the two and half hours normally used with conventional batch well ELISA), which is more than five times faster than the plate method.

The electrochemical system was compared with a commercial spectrophotometric system for the quantification of *H. pylori*-specific IgG antibody in serum samples. The slopes obtained were reasonably close to 1, indicating a good correspondence between the two methods (Fig. 5). Compared with the commercial ELISA test kit, our method shows large enhancement in sensitivity. These results suggest that the detectable concentration of *H. pylori*-specific IgG antibodies in this system is at the levels of clinical analysis, and the sensitivity has reached to the levels to meet the determination *H. pylori*-specific IgG antibodies in serum even in patients with very low levels.

4. Conclusions

In this study a microfluidic enzyme-linked-immunomagnetic assay coupled with electrochemical detection was developed for a rapid, sensitive and selective quantification of IgG antibodies specific against *H. pylori* in human serum sample. The integration of a microfluidic device based on the use of modified paramagnetic microbeads, with a gold electrode to measure an electrical signal, increased the capability to determinate the low levels of IgG antibodies specific to *H. pylori* with high sensibility. The increased reactive surface area and the reduced diffusion distances in our immunoaffinity microfluidics devise permitted a faster time of analysis (25 min) and a less sample consumed than conventional immunoassay techniques. Owing to the wider applications in many fields, miniaturized magnetic immunosensors will make a significant contribution to faster, direct, and secure analysis in many fields as clinical, environmental and food determinations.

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